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# Competitive Androgen Receptor Antagonism as a Factor Determining the Predictability of Cumulative Antiandrogenic Effects of Widely Used Pesticides

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Abbreviations:

AR – Androgen Receptor

CA – Concentration Addition

DDE – 1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene

DHT – dihydrotestosterone

IA – Independent Action

IC01, IC10, IC20, IC50 – Concentrations that inhibits the androgenicity of DHT by 1, 10, 20 or 50%.

**Abstract**

**Background:** Many pesticides in current use have recently been revealed as *in vitro* androgen receptor (AR) antagonists, but information about their combined effects is lacking.

**Objective:** To investigate the combined effects and the competitive AR antagonism of pesticide mixtures.

**Methods:** We tested a combination of 8 AR antagonists that did not also possess AR agonist properties ("pure" antagonists; 8 mix: fludioxonil, fenhexamid, *ortho*-phenylphenol, imazalil, tebuconazole, dimethomorph, methiocarb, pirimiphos-methyl), a combination of 5 AR antagonists that also showed agonist activity (5 mix: cyprodinil, pyrimethanil, vinclozolin, chlorpropham, linuron), and all pesticides combined (13 mix) using the MDA-kb2 assay. Concentration addition (CA) and independent action (IA) were used to formulate additivity expectations and Schild-plot analyses were conducted to investigate competitive AR antagonism.

**Results:** A good agreement between the effects of the mixture of 8 "pure" AR antagonists and the responses predicted by CA was observed. Schild plot analysis revealed that the 8 mix acted by competitive AR antagonism. However, the observed responses of the 5mix and the 13 mix fell between the prediction window defined by CA and IA. Schild plot analysis with these mixtures yielded anomalous responses incompatible with competitive receptor antagonism.

**Conclusions:** For the first time we demonstrate that a mixture of widely used pesticides can produce combined AR antagonist effects that exceed the responses elicited by the most potent component alone in a predictable manner. Considering that large populations are regularly exposed to mixtures of antiandrogenic pesticides, our results underline the need for considering combination effects for these substances in regulatory practice.

**Introduction**

Certain pesticides are known to cause disruption of male sexual differentiation *in vivo* by antagonising the androgen receptor (AR: Gray et al. 1994; Lambright et al. 2000; Ostby et al. 1999) or by interfering with steroid-converting enzymes in fetal life (Blystone et al. 2007; Vinggaard et al. 2005). These pesticides can act together to produce combination effects (Christiansen et al. 2008; Vinggaard et al. 2005), which can also occur in combination with other chemicals known to disrupt androgen action (Rider et al. 2008; Rider et al. 2009). Data from food residues indicate that there is a potential for human exposure to at least some of these pesticides simultaneously.

We previously reported that a number of current-use pesticides are antiandrogenic (Orton et al. 2011). Using these data we formulated mixtures based on the most common pesticides present in foods in Europe. Many of these pesticides are also commonly found in the US (e.g., fludioxonil: 26% of strawberries/14% of grapes; fenhexamid: 24% of strawberries; ortho-phenylphenol: 34% of oranges; dimethomorph: 28% of lettuces; cyprodinil: 27% of grapes; pyrimethanil: 31% of strawberries; chlorpropham: 76% of potatoes: EPA 2011). Considering that risk assessment procedures do not take account of mixture effects at present, it is possible that risks to male reproductive health by pesticides are being underestimated. Although antiandrogenic mixture effects have been described for certain pesticides, some of them obsolete (Nellemann et al. 2003, Kjærstad et al. 2010, Birkhoj et al. 2004), similar data with more widely used pesticides are lacking. Since it is known that many current-use pesticides are AR antagonists *in vitro* (Kojima et al. 2004; Orton et al. 2009; Orton et al. 2011), it is plausible to assume that there might also be mixture effects of these pesticides. However, empirical evidence to support this idea is missing. Because none of the pesticides chosen for our mixture studies were tested *in vivo*, it was important to investigate whether these substances have the ability to act jointly at the receptor level *in vitro*. If that was found to be the case, it would create alerts for prioritisation for *in vivo* testing.

This is all the more relevant as there are indications of negative effects on male reproductive health from epidemiological studies of occupational pesticide exposures. For example, statistically significant associations between genital malformations/penile length in boys and occupational maternal/paternal pesticide exposure have been observed in the Netherlands (Pierik et al. 2004), Denmark (Andersen et al. 2008; Wohlfahrt-Veje et al. 2012), France (Gaspari et al. 2011) and also in a meta-analysis of hypospadia incidence from several countries (Rocheleau et al. 2009). However, these studies could not pinpoint specific pesticides as being involved in the analysed effects.

At present, the number of registered active ingredients in pesticide formulations in the US is 1252 (USEPA pers. comm.). In Europe there are 411 registered entities, with another 72 "pending" (EC 2011). With such a high number of registered active substances, it is practically impossible to test all possible combinations to arrive at robust conclusions about the nature of combination effects. Therefore, exploring the accurate predictability of mixture responses using modelling approaches is essential. Mixture modelling utilises single compound testing data in order to describe the effects of simultaneous exposures to multiple chemicals, with the aim of replacing or significantly reducing testing for the prohibitively large number of chemicals and combinations present in the environment. In this context, modelling approaches work under the hypothesis that compounds elicit their effects without influencing the toxicity of other mixture components, the additivity assumption (for review see: Kortenkamp 2007). Two concepts are commonly used to explore the additivity assumption: Concentration addition (CA, also called Dose Addition) and independent action (IA, also called Response Addition). CA assumes that all compounds have a similar mechanism of action (e.g., binding the same receptor), whereas IA presumes that all mixture components affect the same endpoint via different sites or modes of action (dissimilar action). Both additivity models assume that there is no interaction between the compounds, neither on a physico-chemical level nor in their toxicokinetics and toxicodynamics.

CA has consistently been shown to be a good model for predicting antiandrogenic effects, for example, *in vivo* (Christiansen et al. 2008; Hass et al. 2007; Howdeshell et al. 2008) and *in vitro* (Ermler et al. 2011).

To our knowledge, there are only two examples where CA failed to predict the mixture effect. A significant deviation (synergism) was observed in response to 5 anti-androgenic parabens *in vitro* (Kjaerstad et al. 2010) and to 4 anti-androgenic contaminants *in vivo* (di(2-ethylhexyl) phthalate, two fungicides present in food, vinclozolin and prochloraz and a pharmaceutical, finasteride: Christiansen et al. 2009). To investigate the predictability of mixtures of AR antagonists using the MDA-kb2 assay, and considering the features of this assay, we hypothesised that CA, and not IA, would be the appropriate prediction concept (for an overview see: Ermler et al. 2011).

It is known that some AR antagonists possess the ability to also stimulate the receptor, sometimes at concentrations higher than those required for antagonism, in other cases over the same concentration range (Ermler et al. 2011; Orton et al. 2011). Many AR antagonists are not capable of eliciting AR agonist effects, and these are referred to as "pure" antagonists. The antagonist/agonist activity of some antiandrogens is thought to be due to different actions on the AR receptor, whereby the AR is simultaneously stimulated by binding to a distinct domain of the receptor (Tamura et al. 2006). However, it is not known how such effects could affect the predictability of mixture models and if the "similarity" criterion of CA is fulfilled under these circumstances. Therefore we investigated if CA was a suitable prediction tool for mixtures regardless of mixture composition; or if mixtures composed of antagonist/agonist antiandrogens produced responses that deviated from CA. We used a Schild plot analysis to distinguish the similarity requirements for both scenarios. This is a pharmacological method which allowed us to assess if the antiandrogenic activity observed was solely due to competitive antagonism of DHT binding to the ligand binding domain of the AR (Kenakin 1993).

## Methods

*Test compound selection*

We previously showed that 24 current use and environmentally relevant pesticides were AR antagonists (Orton et al. 2011) and our mixture selection was based on these data. For the 24 that were anti-androgenic, we ranked the pesticides by their Environmental Relevance Ratio (ERR), a measure of combined potency and prevalence, and excluded those with lapsed registration status (as of January 2010) and cytotoxicity at ≤ 10 μM. Twelve pesticides fulfilled these criteria, in order of ERR: dimethomorph (ERR = 45.6; re-registration date = 09.2017), fludioxonil (31.2; 10.2018), fenhexamid (11.9; 12.2015), imazalil (9.9; 12.2021), linuron (6.9; 12.2013), *ortho*-phenylphenol (6.1; 12.2019), pirimiphos-methyl (5.5; 09.2017), tebuconazole (5.5; 08.2019), chlorpropham (2.9; 06.2015), methiocarb (2.5; 09.2017), cyprodinil (2.2; 04.2017) and pyrimethanil (1.0; 05.2017). In addition, vinclozolin was included due to its high ERR (79.8), known *in vivo* potency (e.g. Gray et al. 1994) and continued detection in foodstuffs in Europe (0.38% in 2008 (EFSA 2010) and 0.2% in 2009 (EFSA 2011)), despite its expired registration status (01.2007).

*Chemicals*

Dihydrotestosterone (DHT; > 97% purity) was purchased from Steraloids Ltd. (Croydon, Surrey, UK); dimethomorph and methiocarb were purchased from Greyhound Chromatography and Allied Chemicals (> 98.7% purity; Birkenhead, Merseyside, UK); and all other pesticides (> 97% purity) were purchased from Sigma Aldrich (Poole, Dorset, UK). Ethanol (> 99.7% purity) was obtained from VWR International Ltd. (Leicestershire, UK). All test compounds were dissolved in ethanol to make stock solutions to be used in the assays.

*MDA-kb2 assay.*

MDA-kb2 cells are human breast cancer cells stably transfected with a firefly luciferase reporter gene that is driven by an androgen-response element–containing promoter (American Tissue Culture Collection, ATCC, Wilson et al. 2002). Details of the modified assay were published previously (Ermler et al. 2010). Briefly, cells were seeded at a concentration of $1 \times 10^5$ cells/ml in phenol red–free Leibowitz-15 medium (Invitrogen Ltd., Paisley, UK) containing 10% (charcoal-stripped) fetal calf serum (Invitrogen Ltd.) in white luminometer plates. After 28 hours, luciferase activity was determined with SteadyGlo assay reagent (Promega UK Ltd., Southampton, Hampshire, UK) and measured in a plate reader (FLUOstar Optima, BMG Labtech GmbH, Offenburg, Germany). For regression analysis, cells were exposed to eight serial dilutions of selected pesticides with or without DHT (0.25 nM). Subsequently to the initial testing range of 1.17 nM–150 μM, mixtures concentrations were modified to reflect the potency and toxicity of each individual mixture. For Schild plot analysis, cells were co-exposed with eight serial dilutions of DHT (0.009-20 nM) and fixed concentrations of pesticide mixtures (150-6.25 μM), which varied according to individual activity/toxicity of the mixture. For all testing scenarios, the following controls were run on each plate: media, ethanol (0.25%), DHT co-exposure (0.25 nM) and DHT serial dilutions (0.009–20 nM) with ethanol (0.25%), and procymidone (0.005–3.2 μM) with DHT (0.25 nM). All concentrations were tested in duplicate over two plates, each mixture stock was measured at least twice in separate experiments and mixtures were independently tested at least 3 times (new stock solutions, independent experiment); and were also tested by two experimenters. For comparative purposes, luminescence was normalized to DHT alone at the co-exposure concentration (maximum response, 100%) and solvent-only (ethanol) controls (minimum response, 0%).

*Cytotoxicity as a confounding factor*

The MDA-kb assay measures decreases in luminescence of the DHT agonist that occur as a result of receptor antagonism. Since the luminescence signal can also be driven down by cytotoxicity, it is important to distinguish antagonism from interfering cytotoxicity. We have adopted well established procedures (Korner et al. 2004, Ermler et al. 2010, Ermler et al. 2011) to deal with this issue. Briefly,

cytotoxicity was determined in treatments without DHT by a reduction in luminescence relative to the ethanol controls. Where agonism in the absence of DHT was observed, the comparison was with the maximal response.

*Renilla Assay*

We constructed a *Renilla* luciferase plasmid with a mammalian selection marker and a constitutively active promoter (HSV-TK) in order to eliminate the possible interfering effects of cell proliferation. Briefly, 4 µg DNA was incubated with 6 µl turbofect (Fermentas Gmbh, Germany) in 400 µl of serum-free Leibowitz L-15 media for 20 minutes. MDA-kb2 cells were transfected with the *Renilla* construct for 48 hours prior to following the normal procedure for the MDA-kb2 assay. In order to read both the luciferase and *Renilla* signals, after 28 hours, luciferase activity was determined with Dual-Glo Reporter assay reagent (Promega UK Ltd., Southampton, Hampshire, UK) which employs the sequential addition of two reconstituted reagents with luminescence measurement after each reagent addition (FLUOstar Optima, BMG Labtech GmbH, Offenburg, Germany). The first reagent provides the necessary substrate for *Firefly* luciferase, and the second reagent quenches this activity, while at the same time activating *Renilla* luciferase. Cells transfected with the *Renilla* construct were exposed to the 5 mix IC10 only, for regression analysis 5 mix (serial dilutions: 150-5.6 µM) was co-exposed with a fixed concentration of DHT (0.25 nM) and for Schild plot analysis, serial concentrations of DHT (0.009-20 nM) with various fixed concentrations of 5mix (110-13.75 µM).

*Test mixtures*

All mixtures were designed as fixed ratio equipotent mixtures. We tested three distinct pesticide mixtures, 8 “pure” AR antagonists (8 mix: fludioxonil, fenhexamid, *ortho*-phenylphenol, tebuconazole, dimethomorph, imazalil, methiocarb, pirimiphos-methyl), 5 antagonists with additional agonist properties (5 mix: cyprodinil, pyrimethanil, vinclozolin, chlorpropham, linuron) and the 8 “pure” antagonists with the 5 “mixed” antagonists (13 mix). Fixed-mixture ratios were calculated in proportion to the

concentrations of the individual mixture components that led to a suppression of DHT effects by 1%, 10%, 20% or 50% (here termed inhibitory concentrations IC01, IC10, IC20, IC50). The 13mix was tested at four fixed mixture ratios (IC50, IC20, IC10, IC01) and the 8 mix and 5 mix were tested at two fixed mixture ratios (IC01, IC10) (See Supplemental Material, Table S1). The mathematical and statistical procedures used for calculating mixture effects according to CA and IA are described in Faust et al. (2001).

*Schild plot calculations*

In order to confirm applicability of the MDA-kb2 assay to Schild plot analysis, we first determined concentration-effect curves for the agonist DHT in the presence of various concentrations of flutamide. From the concentration-effect curves, we estimated a series of concentration ratios (the ratio of the DHT concentration to produce a specific effect in the presence of the antagonist to the concentration required in the absence of the antagonist) for a given effect. This was determined for several concentrations of the antagonists. To get the most accurate results, we used a 50% inhibition, which is the concentration ratio was calculated as the IC50 in the presence of antagonist divided by the IC50 in the absence of antagonist. The Schild plot analysis was then based on the linear regression:

$$\log(IC_{50}^{DHT+A} / IC_{50}^{DHT} - 1) = -\log(K_D) + \theta * \log(c_A) \quad [1]$$

Here, $K_D$ is the (unknown) dissociation constant of the antagonist, $c_A$ the concentration of the antagonist "A" held fixed in the experiments and θ the slope parameter. The unknown parameters θ and $\log(K_D)$ were estimated by ordinary least squares. If the regression is linear with a slope of 1, this indicates that the antagonism is competitive and by definition the agonist and antagonist act at the same sites (Kenakin 1993). It should be noted that the concentration response curves recorded in the presence of a fixed concentration of the antagonist will shift to the right of the DHT curve, with the same maximum response and (generally) the same shape. Therefore we always used the logit model for the data analysis, and

performed Schild regression analysis only when the assumption of similar maximum responses was justified. The same principles were applied to the pesticide mixtures.

*Statistics*

To analyse AR antagonist action, raw luminescence readings were normalised on a plate by plate basis to the means of the positive DHT controls (n=8) and the solvent controls (n=8) which were placed on the same plate. Luminescence readings from pesticides tested in the absence of DHT were divided by the mean of the solvent controls from the same plate and analysed for negative and positive trends (suggestive of cytotoxic or androgenic action, respectively). All data from the same test compound were pooled and statistical concentration response regression analyses were conducted by using the best-fit approach to derive inhibitory concentrations (IC) for androgenicity (Scholze et al. 2001). To control for variations between experiments, concentration response data were analysed by using a generalised non-linear mixed modelling approach (Vonesh and Chinchilli 1996) with plate as a random effect modifier for individual effect data. If readings in the absence of DHT showed indications for cytotoxic or androgenic action, the non-monotonic concentration-response relationship was modelled by non-parametric local regression methods (Cleveland et al. 1988). From this robust fitting method we derived effect concentrations (EC) for androgenicity, with a 10% increase over the mean solvent mean as the minimum effect criterion, and ECs for cytotoxicity (if present) as 10% reduction of the maximal observed androgenic action. Data points associated with cytotoxicity were not included in regression analysis for antiandrogenicity. Differences between predicted and observed effect doses were deemed statistically significant when the 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects. All statistical analyses were performed using the SAS statistical software (SAS Institute Inc., Cary, NC, USA).

**Results**

Low variation between experiments, good repeatability and complete regression curves for all the selected individual pesticides meant that all compounds were suitable for mixture assessment. All mixtures showed AR antagonist activity in a clear dose-dependent way (Figure 1 A-D). The agreement between observed and predicted AR antagonistic activity for a given mixture is shown for two selected response levels in Table 1: IC50s for the mixtures were only once overestimated by both CA and IA (13 mix, IC01 mixture ratio, 10% inhibition) and in all other cases were never outside the range predicted by CA and IA. Cytotoxicity was only observed at high mixture concentrations (8 mix: EC10 = 60-77 μM; 5 mix: EC10 = 70-74 μM; 13 mix; EC10 = 63-81 μM, see Supplemental Material, Table S2 for more information). The overlap with antiandrogenic responses was negligible and did not interfere with the detection of AR antagonistic responses (Figure 1 A-D). The model parameters, together with estimated AR antagonist concentrations and effect concentrations for androgenicity and cytotoxicity are listed in Supplemental Material, Table S2. Cytotoxicity data for all test mixtures are also shown in Supplemental Material, Figure S2.

There was good agreement of the 8mix responses with those predicted by CA, over the entire concentration range and for both tested mixture ratios (Figure 1A/B, Table 1). This mixture was composed entirely of "pure" AR antagonists. However, CA consistently overestimated the combined effects of mixtures containing AR antagonists that also showed AR agonistic properties (5 mix: Figure 1C; and 13 mix: Figure 1D). With these two mixtures we observed androgenic activity at low concentrations when tested in the absence of DHT (Supplemental Material, Table S1). When tested on their own, none of the individual pesticides in the mixtures showed AR agonistic effects at their concentration in the mixture. The androgenicity of 5 mix and 13 mix therefore appears to be a genuine

combination effect. Indications for toxicity were detected only at high tested concentrations and are unlikely to interfere with the mixture assessment.

By performing Schild plot analysis with the pure antiandrogen flutamide and DHT we were able to confirm competitive receptor antagonism. Increasing concentrations of this antagonist shifted the dose-response curve of the agonist DHT to the left (Supplemental Material, Figure S1A/B), indicating that agonist and antagonist acted in a competitive manner at the same receptor site, the ligand-binding domain of the AR.

We obtained similar results with a Schild plot analysis of the 8mix which was composed of "pure" AR antagonists. Increasing concentrations of the 8 mix shifted the DHT curve progressively towards lower concentrations, without affecting the maximal response of the agonist. The resulting Schild plot was linear which suggests that the observed AR antagonistic effect of the mixture was indeed due to competitive AR antagonism, without being confounded by multiple binding sites or pharmacokinetic interactions (Figure 2A/B).

However, in the presence of the 5 mix and 13 mix the maximal effects observed at saturating DHT concentrations rose far above the levels normally seen with the agonist on its own ("supramaximal" effects) (Figure 2C/E). These supramaximal DHT responses increased with rising mixture concentrations until 100 µM (5 mix) and 70 µM (13 mix). Beyond these concentrations a down-turn of responses was observed (Figure 2D/F). This down-turn corresponded with the cytotoxicity values obtained by analysis of the test mixture in the absence of DHT and thus can be explained in terms of this mechanism. Supramaximal effects violate one basic assumption of the Schild plot analysis, namely that an antagonist should not influence the maximal response of the agonist. For this reason, Schild plots could not be constructed for the 5 mix and 13 mix. These results show that the suppression of DHT effects seen with these two mixtures are not solely due to competitive receptor antagonism and suggest that more complex processes are operational at the receptor.

To investigate if stimulation of the maximal response with the 5 mix and DHT was the result of cell proliferation, we utilised a *Renilla* luciferase construct in our assay. This construct produces luminescence in proportion to cell number, independent of AR activation. There was no dose-response relationship between rising concentrations of the 5 mix and *Renilla* luminescence of the MDA-kb2 cells and no differences in luminescence between ethanol only (mean ± STD: 3335 ± 896) and the DHT background concentration only (3036 ± 756). The same applied to DHT (3059 ± 689), the positive control procymidone (4115 ± 820) and to any concentration of the 5mix with DHT (110 μM: 2198 ± 418; 55.5 μM: 1909 ± 399; 27.5 μM: 2080 ± 359; 13.75 μM: 2340 ± 379) and the 5mix on its own (150-5.6 μM: 2134 ± 322) (data not shown). This indicates that cell proliferation was not the cause of the increased luminescence observed with this mixture and DHT, and that the supramaximal responses were the consequence of phenomena at the receptor.

**Discussion**

For the first time, we demonstrate that a mixture of widely used pesticides can produce combined AR antagonist effects that exceed the responses elicited by the most potent component alone. Furthermore, these mixture effects occurred in a quite predictable manner. The responses of the 8 mix composed of "pure" AR antagonists agreed very well with the combined effects predicted by CA. However, the combined effects seen with the two mixtures containing AR antagonists that also showed AR agonist properties (5 mix and 13 mix) were somewhat lower than anticipated by CA and fell in the "prediction window" defined by CA and IA. These deviations are highly unlikely due to experimental artefacts because the mixtures were tested independently by different experimenters who prepared several independent mixture stock solutions.

By conducting Schild plot analysis we were able to pinpoint competitive AR antagonism as a key factor that influenced agreement of the experimentally observed responses with the CA predictions. The combination of 8 "pure" AR antagonists (8 mix), well predicted by CA, produced Schild plots typical of competitive receptor antagonism. In contrast, anomalous supramaximal effects were observed with the 5mix and 13mix in experiments where increasing concentrations of these two mixtures were combined DHT. These anomalies suggest that the 5 mix and 13 mix displaced DHT from the AR in ways not compatible with competitive antagonism. This allows us to infer that the lack of competitive AR antagonism is the likely cause of the observed deviations from CA. It is known that chemicals which display mixed androgenic/antiandrogenic activity interact in ways with the amino acid residues in the AR binding domain that are distinct from those of "pure" AR antagonists (Tamura et al. 2006). It has also been shown that chemicals containing pyrimidine domains such as cyprodinil and pyrimethanil, can cause AR antagonism via a non-ligand binding domain of the AR (Gunther et al. 2009). Pesticides of this kind formed a large proportion of the 5 mix and 13 mix, but were not present in the 8 mix. Although further mechanistic studies would be necessary to substantiate these ideas, we suggest that these modalities may

play a role in the deviations from expected concentration additivity that we observed with the 5 mix and 13 mix. However, other explanations, such as stabilization of the AR-DNA binding complex or downstream effects of the signalling pathway may also be valid. In addition, there is some evidence that estrogenic supramaximal effects may be assay specific (Montaño et al. 2010), and although similar data are not available for AR antagonist assays, this is also a possible explanation for observed effects.

Deviations from expected additivity are interesting from a mechanistic viewpoint, but their relevance in relation to the application of CA or DA in risk assessment practice is not well defined. While it is reasonable that similarly acting pollutants should be assessed together, it is a matter for debate how chemicals should be grouped that do not match very narrowly defined criteria for similarity. Specifically, the question raised by our results is whether only "pure" AR antagonists that displace DHT in a competitive manner qualify for inclusion in groupings conforming to CA, and whether therefore AR antagonists with AR agonistic properties should be excluded from joint assessments under CA principles. In resolving this issue it is helpful to consider how combinations of AR antagonists behave *in vivo*. The applicability of CA for mixtures composed of AR antagonists was tested in a rat developmental toxicity model with flutamide, procymidone ("pure" antagonists) and vinclozolin (which liberates metabolites that possess mixed AR antagonistic and agonistic properties) (Hass et al. 2007; Metzdorff et al. 2007). In these studies, the observed responses, including ano-genital distance, reproductive organ weights and prostate gene expression (PBP C3) did not differ significantly from CA. For nipple retention, the observed response slightly exceeded the predicted mixture effects (Hass et al. 2007). Although it is not possible to arrive at firm conclusions based on these studies, it appears that the AR antagonist and agonist properties observed *in vitro* do have negligible impacts on the effects that are observed *in vivo*. A recent report has recommended that despite the small deviations from CA that have sometimes been observed *in vitro* and *in vivo*, the evidence overwhelmingly suggests that it is a more accurate prediction model than IA (Kortenkamp et al. 2012). Furthermore, CA is a more conservative estimate of effect than IA and thus CA would be protective for mixtures that fall in the prediction window (Kortenkamp et al. 2012).

Therefore, we propose that CA is a suitable model for mixtures that contain AR antagonists with agonist properties, and that these chemicals should be grouped together with "pure" AR antagonists.

Early life exposure is thought to be crucial for the development of abnormalities in male reproductive health (Skakkebaek et al. 2001). Fresh fruit and vegetables are consumed in high amounts by women and young children (Claeys et al. 2010), but these food items contain both the highest concentrations of single pesticides and the highest percentage with multiple residues (EFSA 2010; 87%: Inigo-Nunez et al. 2011; 90%: Schiliro et al. 2011). It is also interesting to note that a strong association between hypospadias and consumption of market fruit (OR(CI): 5.10(1.31-19.82) was reported from an agricultural population of Italy (Giordano et al. 2008). Fungicides were the most common pesticides detected in fruits and vegetables in several studies (Claeys et al. 2010; Inigo-Nunez et al. 2011; Schiliro et al. 2011) and they make up 9 of the 13 pesticides selected here for testing. Although fungicides have broadly comparable use, as indicated by their global market share (22%), compared to herbicides (40%) and insecticides (29%) (Grube et al. 2011), fungicides are often applied post-harvest. As a result, their contribution to human exposures may well be higher than that of other pesticides. Due to rapidly evolving resistance of target organisms to fungicides, they are recommended to be applied in mixtures for maximum effectiveness (FRAC 2010). For example, the commercial formulation "switch" contains cyprodinil and fludioxonil (Syngenta 2011), "forum" is composed of dithianon and dimethomorph (BASF 2010), "Justmeet" of fenhexamid and fludioxonil, and "Teldor Combi" contains fenhexamid and tebuconazole (Bayer 2011). There is a clear potential for human exposures via residues on foodstuffs, but to our knowledge, human biomonitoring data for the fungicides tested in this study are not available. There is also a lack of *in vivo* data for pesticides tested in this mixture (see: Orton et al. 2011). For these reasons, it is currently not possible to ascertain the relationship between *in vitro* potency, *in vivo* effects and exposure with adequate certainty, but such information is required if the risks to human health are to be properly assessed.

**Conclusions**

For the first time we demonstrate that widely used pesticides act additively *in vitro* as AR antagonists. The less accurate predictability of mixtures containing antagonists that also have agonist activity may due to distinct action at the ligand-binding domain of the AR. Despite the unknown pharmacological cause of deviation from CA, it should still be used for risk assessment due to the minimal deviation observed and the protective (worst-case) nature of CA. It is well known that people are exposed to mixtures of pesticides, and therefore, the additive nature of these pollutants is a cause for concern.

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**Table 1: Statistical uncertainty of predicted and observed inhibitory concentrations for mixtures**

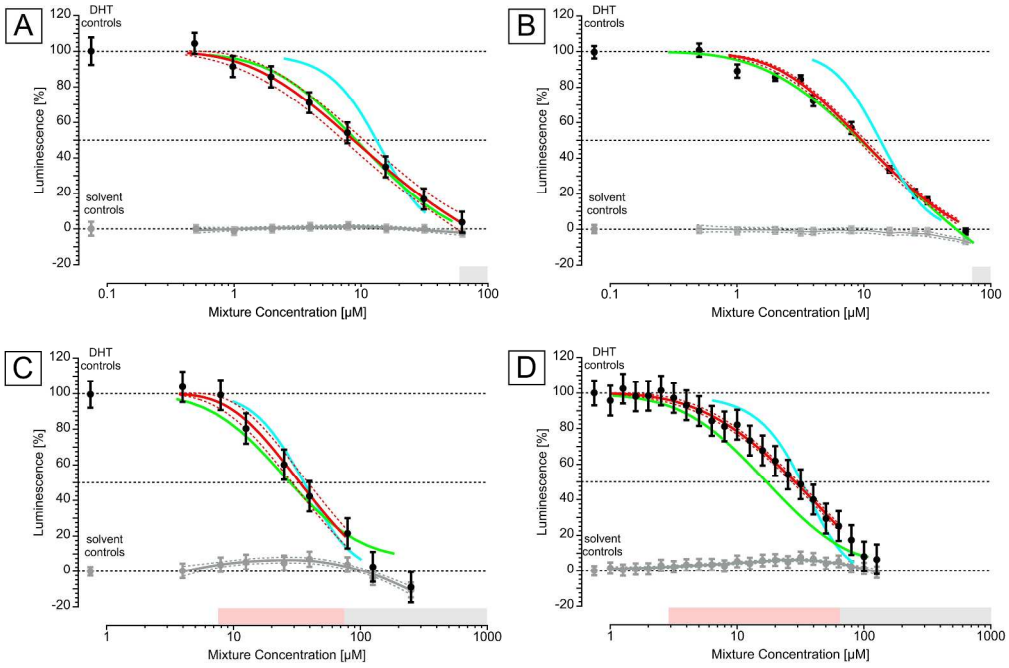
| Inhibition level *x* | Inhibition concentration $IC_X$(mixture) [M] | | | | | |
|---|---|---|---|---|---|---|
| | Observed | | Predicted by CA | | Predicted by IA | |
| | **Mean** | **95% CI** | **Mean** | **95% CI** | **Mean** | **95% CI** |
| Eight pesticides (IC01 ratio)[a] | | | | | | |
| 10 % | 1.65E-6 | (1.11E-6, 2.21E-6) | 2.12E-6 | (1.87E-6, 2.29E-6) | 5.47E-6 | (4.10E-6, 6.18E-6)* |
| 50 % | 9.74E-6 | (7.91E-6, 1.07E-5) | 9.66E-6 | (9.00E-6, 1.03E-5) | 1.35E-5 | (1.20E-5, 1.46E-5)* |
| Eight pesticides (IC10 ratio)[a] | | | | | | |
| 10 % | 1.49E-6 | (1.23E-6, 1.85E-6) | 1.87E-6 | (1.64E-6, 2.03E-6) | 4.57E-6 | (3.46E-6, 5.26E-6)* |
| 50 % | 8.79E-6 | (7.55E-6, 1.04E-5) | 9.13E-6 | (8.53E-6, 9.76E-6) | 1.32E-5 | (1.17E-5, 1.45E-5)* |
| Five pesticides (IC01 ratio)[a] | | | | | | |
| 10 % | 1.03E-5 | (8.25E-6, 1.21E-5) | 6.73E-6 | (5.86E-6, 7.82E-6)* | 1.39E-5 | (1.13E-5, 1.66E-5) |
| 50 % | 3.35E-5 | (2.95E-5, 3.78E-5) | 2.80E-5 | (2.58E-5, 3.09E-5) | 3.60E-5 | (3.28E-5, 4.10E-5) |
| Five pesticides (IC10 ratio)[a] | | | | | | |
| 10 % | 8.97E-6 | (6.77E-6, 1.20E-5) | 6.38E-6 | (5.47E-6, 7.28E-6) | 1.33E-5 | (9.93E-6, 1.67E-5) |
| 50 % | 3.54E-5 | (3.11E-5, 4.06E-5) | 2.89E-5 | (2.68E-5, 3.19E-5) | 3.77E-5 | (3.41E-5, 4.32E-5) |
| 13 pesticides (IC01 ratio) [a] | | | | | | |
| 10 % | 5.56E-6 | (4.38E-6, 7.39E-6) | 3.89E-6 | (3.55E-6, 4.16E-6)* | 1.38E-5 | (1.06E-5, 1.52E-5)* |
| 50 % | 2.61E-5 | (2.38E-5, 2.95E-5) | 1.70E-5 | (1.61E-5, 1.80E-5)* | 3.11E-5 | (2.83E-5, 3.37E-5) |
| 13 pesticides (IC10 ratio)[a] | | | | | | |
| 10 % | 5.20E-6 | (3.47E-6, 7.28E-6) | 3.61E-6 | (3.24E-6, 3.88E-6) | 1.11E-5 | (8.24E-6, 1.27E-5)* |
| 50 % | 2.86E-5 | (2.68E-5, 3.01E-5) | 1.71E-5 | (1.63E-5, 1.80E-5)* | 3.14E-5 | (2.81E-5, 2.81E-5) |
| 13 pesticides (IC20 ratio)[a] | | | | | | |
| 10 % | 3.25E-6 | (2.28E-6, 4.91E-6) | 3.48E-6 | (3.14E-6, 3.75E-6) | 1.01E-5 | (7.30E-6, 1.17E-5)* |
| 50 % | 2.42E-5 | (2.09E-5, 2.89E-5) | 1.71E-5 | (1.63E-5, 1.80E-5)* | 3.06E-5 | (2.72E-5, 3.35E-5) |
| 13 pesticides (IC50 ratio)[a] | | | | | | |
| 10 % | 5.41E-6 | (3.70E-6, 7.44E-6) | 3.35E-6 | (2.99E-6, 3.64E-6)* | 9.14E-6 | (6.47E-6, 1.08E-5) |
| 50 % | 3.24E-5 | (2.70E-5, 3.67E-5) | 1.75E-5 | (1.65E-5, 1.85E-5)* | 2.89E-5 | (2.57E-5, 3.20E-5) |

CA – Concentration Addition, IA – Independent Action, CI – Confidence Interval; All predictions statistically significant to the observed ICs are indicated by an asterisks. Significance between predicted and observed $IC_X$ values was judged as a non-overlapping of their 95% percentile bootstrap confidence intervals. [a] See Supplemental Material, Table S1 for definitions.

**Figure Legends**

**Figure 1**: Predicted and observed anti-androgenic activity of mixtures with 8 pesticides composed in the ratio of their individual IC10 values (A) and IC01 values (B), with 5 pesticides mixed in the ratio of their individual IC01 values (C), and with 13 pesticides mixed in the ratio of their individual IC10 values (D). Observed mixture effects are from at least three independent mixture experiments and shown as mean ± standard deviation, predicted effect curves were calculated using the model of CA (solid green line) and IA (solid blue line). Regression fit of the observed effects is shown as red line, with the dotted red line indicating the corresponding 95% confidence belt. In addition, mean responses ± standard deviation are shown for the tested mixture without DHT (gray), together with the estimated mean effect (solid gray line) and 95% confidence belt (dotted gray line). Androgenicity is highlighted as red bar above the concentration scale, cytotoxicity as grey bar.

**Figure 2**: Anti-androgenic activity of DHT in the presence of fixed mixture concentrations of 8 pesticides (IC10 mixture ratio, A), 5 pesticides (IC01 mixture ratio, C) and 13 pesticides (IC01 mixture ratio, E). For the mixture of 8 pesticides the Schild regression plot is shown (B), for the 5 and 13 component mixtures the estimated maximal effect levels (± 95% confidence belt) in dependence of the fixed mixture concentrations (D and F, respectively).

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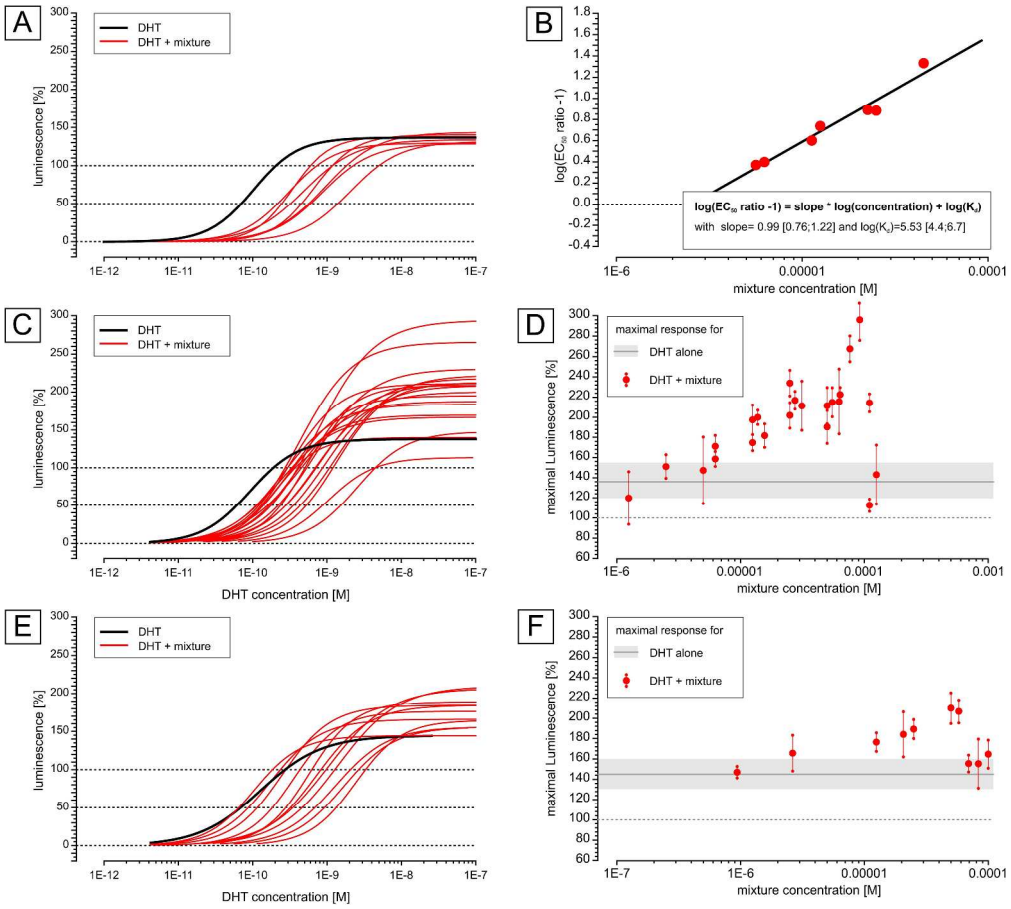


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